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Hereditary Pancreatitis Caused by a Novel PRSS1 Mutation (Arg-122 → Cys) That Alters Autoactivation and Autodegradation of Cationic Trypsinogen*

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Hereditary pancreatitis has been found to be associated with germline mutations in the cationic trypsinogen (PRSS1) gene. Here we report a family with hereditary pancreatitis that carries a novel PRSS1 mutation (R122C). This mutation cannot be diagnosed with the conventional screening method using *Afl*III restriction enzyme digest. We therefore propose a new assay based on restriction enzyme digest with *Bst*UI, a technique that permits detection of the novel R122C mutation in addition to the most common R122H mutation, and even in the presence of a recently reported neutral polymorphism that prevents its detection by the *Afl*III method. Recombinantly expressed R122C mutant human trypsinogen was found to undergo greatly reduced autoactivation and cathepsin B-induced activation, which is most likely caused by misfolding or disulfide mismatches of the mutant zymogen. The K_m of R122C trypsin was found to be unchanged, but its k_{cat} was reduced to 37% of the wild type. After correction for enterokinase activatable activity, and specifically in the absence of calcium, the R122C mutant was more resistant to autolysis than the wild type and autoactivated more rapidly at pH 8. Molecular modeling of the R122C mutant trypsin predicted an unimpaired active site but an altered stability of the calcium binding loop. This previously unknown trypsinogen mutation is associated with hereditary pancreatitis, requires a novel diagnostic screening method, and, for the first time, raises the question whether a gain or a loss of trypsin function participates in the onset of pancreatitis.

Hereditary pancreatitis was initially reported as a form of chronic pancreatitis that is clinically indistinguishable from other etiological varieties of the disease but is inherited as an autosomal dominant trait (1). Five years ago the genetic basis of the disease was firmly established when a germline mutation in the cationic trypsinogen gene (PRSS1) was found to associate with the disease phenotype (2). In most cases the disease begins with recurrent episodes of acute pancreatitis in children and young adults and progresses to chronic pancreatitis with exocrine and endocrine pancreatic insufficiency (3). The penetrance of the most common trypsinogen mutations is 80%, and, for yet unknown reasons, unaffected mutation carriers neither develop pathological changes in the pancreas nor share the increased pancreatic cancer risk of their affected relatives (4, 5). The underlying pathophysiological mechanisms through which carriers of trypsinogen mutations develop pancreatitis are unknown. The most intuitive explanation would be that either one of these mutations leads to a gain of trypsin function, *i.e.* a more rapid or efficient intrapancreatic trypsinogen activation or an extended activity of trypsin resulting from impaired inactivation or autolysis (6–10). Trypsin would then, in analogy to the conditions in the small intestine, activate other digestive proteases in a cascade-like fashion and thus mediate acinar cell injury (11). Here we report a family with hereditary pancreatitis that differs from previously reported kindreds in several respects. 1) Affected patients carry a previously unreported Arg-122 → Cys mutation that cannot be detected with the conventional screening technique based on an *Afl*III restriction enzyme digest. 2) The penetrance of the disease phenotype appears to be lower than that of the most common PRSS1 mutations. 3) Molecular modeling suggests that this mutation does not impair the active site of cationic trypsin but could affect the calcium binding site of the molecule. 4) Recombinant Arg-122 → Cys trypsin was found to be more resistant to autoactivation as well as to autodegradation under defined experimental conditions and therefore raises the question whether a gain of trypsin function or a loss of trypsin function is involved in the onset of hereditary pancreatitis.

MATERIALS AND METHODS

Nomenclature—All data refer to the human cationic trypsinogen gene or protein (PRSS1 gene, GenBank™ accession no. U66061). To denote PRSS1 mutations, the chymotrypsinogen amino acid numbering system has been used in the past. Because genetic alterations in the PRSS1 sequence have been observed for which no corresponding amino acid exists in the chymotrypsinogen sequence, this practice has been abandoned and the actual human PRSS1 sequence is now used by convention (9). The previously named Asn-21 → Ile mutation is now referred to as Asn-29 → Ile, and the previous Arg-117 → His has become Arg-122→His. In this report we only use the new nomenclature with single-letter abbreviations (*e.g.* N29I, R122H).

Kindred with Hereditary Pancreatitis—A 54-year-old patient was admitted for recurrent episodes of pancreatitis and changes consistent with chronic pancreatitis on endoscopic retrograde cholangio-pancre-

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atography and computed tomography. His family originates from a region of Germany where hereditary pancreatitis is fairly common and over 20 extended kindreds have so far been identified. When his 24-year-old son was found to also suffer from recurrent episodes of pancreatitis and no other risk factors could be identified in both patients' history and clinical evaluation, informed consent was obtained for genetic testing. After both patients were found to carry a previously unreported mutation in the cationic trypsinogen gene, additional informed consent for anonymous testing was obtained from other first degree relatives (see pedigree in Fig. 1).

Genetic Testing, DNA Sequencing, and PCR/Restriction Fragment Length Polymorphism—Leukocyte DNA was extracted from EDTA blood samples using the QIAamp DNA blood kit (Qiagen, Düsseldorf, Germany). DNA was stored in Tris/EDTA buffer, and exon 3 of the cationic trypsinogen was amplified by polymerase chain reaction using specific primers Ex3s (GGTCCTGGGTCTCATACCTT) and Ex3as (GGGTAGGAGGCTTCACACTT). Routine screening for the most common R122H mutation was carried out by *Afl*III restriction endonuclease digest at 37 °C (2). This endonuclease recognizes a novel site created by the R122H mutation. When the R122H mutation was not detected in either patient, all five trypsinogen exons were amplified by PCR using specific primers, subsequently sequenced, and the results compared with the published PRSS1 sequence. For sequencing, the PerkinElmer Big Dye sequencing kit and an ABI Prism 7700 Sequencer were used. A restriction site analysis was undertaken to identify restriction enzymes that would permit to establish a rapid screening method that detects the novel mutation. *Bst*UI was found to be an appropriate enzyme for this purpose, and *Bst*UI restriction endonuclease digestion was subsequently performed at 60 °C. *Bst*UI recognizes a single restriction site in the wild-type sequence of exon 3 that is destroyed by the R122H as well as the R122C mutation.

Recombinant Expression of Cys-122 Trypsin—Ecotin was overexpressed in *Escherichia coli* BL21 (DE3) as described by Pál *et al.* (12, 13) and purified to homogeneity using a trypsin affinity column. Purified ecotin was immobilized to Actigel ALD resin (Sterogene Bioseparations, Carlsbad, CA) as described previously (14). Plasmid pTrap was a generous gift from László Gráf (Eötvös University, Budapest, Hungary) and competent *E. coli* BL21(DE3) cells were purchased from Novagen, Inc. (Madison, WI). Ultrapure bovine enterokinase was purchased from Biozyme Laboratories (San Diego, CA) and *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide from Sigma. Recombinant human cationic trypsinogen cDNA was generated as reported previously (7, 8) and ligated into the modified trypsinogen expression vector pTrap-T7 under the control of the T7 promoter, which allows high level expression in *E. coli* strains expressing T7 RNA polymerase (8). The NH_2-terminal sequence of recombinant human cationic trypsinogen used in this study is Met-Ala-Pro-Phe-Asp-Asp-Asp-Asp-Lys-Ile-, where the Lys-Ile bond is the site of proteolytic activation by enterokinase or trypsin. The DNA sequence of the entire gene was verified by dideoxy sequencing. The Cys-122 mutation was introduced by polymerase chain reaction mutagenesis. Wild-type and Cys-122 cationic trypsinogen were expressed in *E. coli* BL21(DE3) in LB media with 50 μg/ml carbenicillin, grown to an $A_{600\,nm}$ of 0.5, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and grown for an additional 5 h. Inclusion bodies were isolated, and trypsinogens were re-folded and purified via ecotin affinity columns as reported previously (8).

Trypsinogen Activation and Trypsin Autolysis Measurements—Trypsin activity was determined using the synthetic chromogenic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Kinetics of the chromophore release was followed at 405 nm in 0.1 M Tris-HCl, pH 8.0, 1 mM $CaCl_2$, at 22 °C. Autolytic degradation of trypsin was followed by residual activity measurements (15, 16). Wild-type and Cys-122 trypsinogen (final concentration, ~2.5 μM) were activated with 200 ng/ml enterokinase (final concentration) for 60 min at 22 °C in 0.1 M Tris-HCl (pH 8.0), 5 mM $CaCl_2$. Trypsin solutions were then incubated at 37 °C without any further additions (*i.e.* in the presence of 5 mM Ca^{2+}) or after addition of EDTA (pH 8.0) to a final concentration of 10 mM. To study the activation of wild-type and Cys-122 trypsinogen by cathepsin B, the latter (human cathepsin B; Calbiochem, San Diego, CA) was first activated with 0.1 mM dithiothreitol on ice for 10 min before 1 unit/ml active cathepsin B was added to a final volume of 50 μM trypsinogen solution (2.0 μM, 0.1 M sodium acetate buffer, pH 5.0, 2 mM $CaCl_2$). At the indicated time intervals in all experiments, 2.5-μl samples were withdrawn for trypsin activity determination. To determine autoactivation, the trypsinogens (final concentration, 2.5 μM) were incubated at 37 °C in 0.1 M Tris-HCl (pH 8.0) or 0.1 M sodium acetate buffer (pH 5.0) in the presence of 5 mM $CaCl_2$ or 1 mM EDTA, in a final volume of 100 μl. At the indicated time intervals, 2.5-μl aliquots were removed for trypsin activity assays. Alternatively, for gel electrophoresis of recombinant trypsinogen, the reaction was terminated by trichloroacetic acid and centrifuged at 14,000 rpm (4 °C) for 10 min. The protein concentrations were determined from their ultraviolet absorbance using a calculated extinction coefficient of 36,160 $M^{-1}\,cm^{-1}$ at 280 nm, and equal amounts of protein were used for 12% SDS-polyacrylamide gel electrophoresis under reducing or nonreducing conditions. Bands were visualized by Coomassie Blue staining. Data in the graphs represent means ± S.E. from three or more experiments in each group.

Molecular Modeling—For modeling studies the program Deep-View version 3.5.1 (17) was used, and the human trypsin crystal structure at 2.2-Å resolution served as a template (Protein Data Bank entry 1trn) as published by Gaboriaud and co-workers (18). The R122C mutation was introduced with the "mutation tool," and the energetically most favorable rotamers were selected by direct visual comparison on a WinNT work station. The wild type and the mutant structures were energy-minimized to a total energy of −11,400 kJ/mol and −9200 kJ/mol, respectively, to which Arg-122 contributed −260 kJ/mol and Cys-122 12 kJ/mol.

RESULTS

The fact that our patient had developed chronic pancreatitis as an adult would not have suggested hereditary pancreatitis as a differential diagnosis, despite the fact that we could not identify any apparent risk factors for pancreatitis such as alcohol abuse or metabolic disorders. After it became apparent that his son also suffered from recurrent episodes of pancreatitis, we investigated the possibility of genetic alterations in the PRSS1 gene. When the most common hereditary pancreatitis-associated PRSS1 mutation (R122H) was not detected by standard *Afl*III restriction enzyme digest, the entire coding sequence of the PRSS1 gene was sequenced. Both patients were found to be carriers of a previously unreported C to T transition mutation at position 133,282 of the published genomic sequence (GenBank™ accession no. U66061; Fig. 1). This mutation results in an arginine to cysteine amino acid substitution in position 122 (R122C) and therefore affects the same codon as the common R122H mutation. Among the first degree relatives of the patients, two more were found to be carriers of the same mutation (aged 25 and 45 years), but neither was aware of an episode of pancreatitis in the past (Fig. 1). In this particular kindred, this would indicate a disease penetrance of 50%, provided that neither of the two unaffected carriers develops pancreatitis later in life. The latter remains a distinct possibility because of the late symptom onset in both affected patients (at age 52 and 24 years, respectively).

The C to T transition eliminates a single *Bst*UI site in the PCR fragment of exon 3, which permits rapid screening for the new R122C mutation by a simple *Bst*UI restriction digest without the need of DNA sequencing.

To compare *Afl*III and *Bst*UI in their ability to recognize clinically relevant trypsinogen mutations, we used PCR to amplify exon 3 from DNA of healthy controls, from known heterozygous carriers of R122H mutation, and from our patients who carried the R122C mutation, and performed restriction digests with both enzymes (Fig. 2). As predicted by the sequence, the *Afl*III digest detected a restriction site in the R122H carrier, but failed to detect the R122C mutation. On the other hand, a complete *Bst*UI digest was found only in the healthy control cDNA, whereas, in carriers of the R122H as well as the R122C mutation, the restriction site on one allele was destroyed and this resulted in the presence of an undigested PCR product in addition to the two restriction fragments of the wild-type allele. As indicated in Fig. 2, the same *Bst*UI digest would also detect the mutation in the presence of a recently reported neutral polymorphism in exon 3 that is not disease-relevant but makes the common R122H mutation inaccessible for screening with *Afl*III (19, 20).

Characteristics of Recombinant Cys-122 Trypsin—Recombinant wild-type and Cys-122 mutant human cationic trypsino-

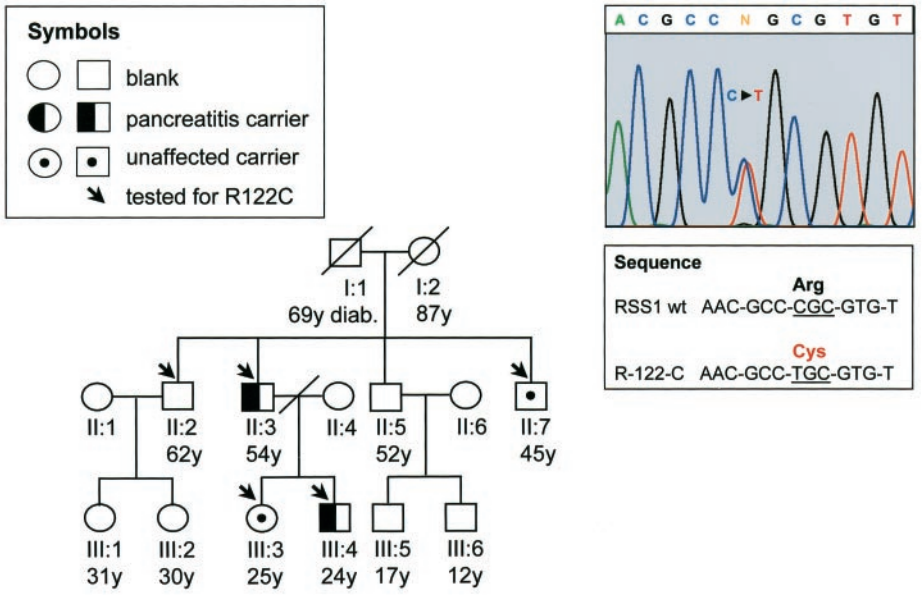

FIG. 1. *Left panels*, pedigree of the family with hereditary pancreatitis and the results of genetic testing for the R122C mutation. Symbol definitions are indicated in the *top left corner*. *Right panels*, electropherogram of the exon 3 sequence indicating the C to T transition in position 133,282 of the wild-type PRSS1 sequence (GenBank™ accession no. U66061) and, *below*, the according amino acid exchange in codon 122.

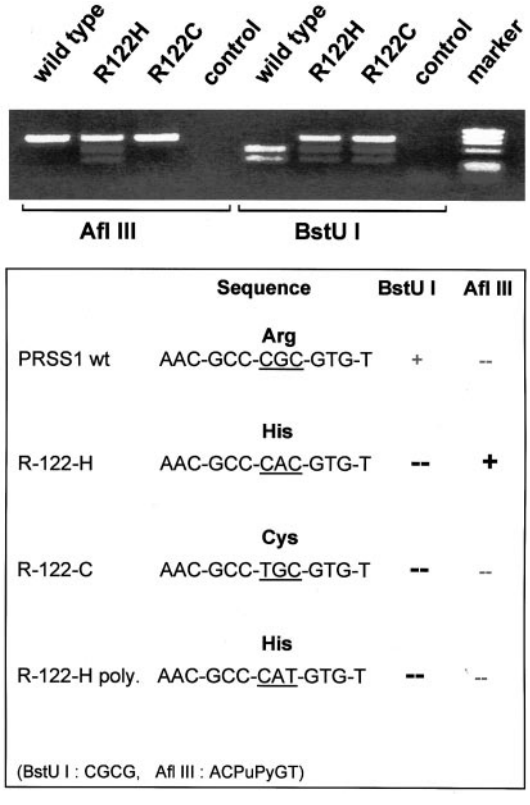

FIG. 2. **Ethidium bromide-stained agarose gel of PCR fragments from wild-type, R122H, and R122C cDNA after restriction enzyme digest with either *Afl*III (*left lanes*) or *Bst*UI (*right lanes*).** Although the *Afl*III digest detects only the common R122H mutation, the *Bst*UI digest also identifies the R122C mutation and would also be predicted to identify a neutral polymorphism in the same codon that has been reported recently (19, 20) to make the R122H mutation undetectable for the *Afl*III restriction enzyme digest (*lower panel*).

gen (PRSS1) were generated and purified on ecotin affinity columns. The full-length sequence as well as the amino acid exchange at position 122 are indicated in Fig. 3. Under reducing conditions both proteins appear to have the appropriate and identical molecular mass (Fig. 3). Under nonreducing conditions, the Cys-122 mutant runs somewhat more slowly, which could indicate that a significant proportion of the mutant protein is present in a different conformation or that disulfide mismatches have formed. When equal protein amounts of wild-type and Cys-122 trypsinogen were activated with bovine enterokinase at pH 8.0 and in the presence of 5 mM Ca^{2+}, only ~40% of activity was generated from Cys-122 trypsinogen as compared with the wild type (end point measurements after 30-min incubation; data not shown). When we calculated the enzyme kinetics for wild-type and Cys-122 mutant trypsin, the K_m values were not different between the two enzymes, but the k_{cat} of Cys-122 trypsin amounted to only 37% of the wild-type (Table I). This suggests that only 37% of the mutant zymogens forms correctly folded active trypsin upon enterokinase activation and that the remaining portion is present in a conformation that renders the protein non-activatable. When equal protein amounts of wild-type and Cys-122 trypsinogen were studied for autoactivation in the presence of 5 mM Ca^{2+} at pH 8.0 or at pH 5.0, only 20% or less of the activity of wild-type trypsinogen was found for the Cys-122 mutant (Fig. 4, *A* and *B*). When, on the other hand, autoactivation of the two trypsinogens was compared in fractions that were corrected for potential activity (normalized for enterokinase activatable trypsin activity), the results were rather different. In the presence of 5 mM Ca^{2+}, autoactivation of Cys-122 trypsinogen proceeded only slightly faster than wild-type trypsinogen at either pH 5.0 or at pH 8.0 (Fig. 4, *C* and *D*). It must remain open whether this difference is biochemically meaningful in view of the wholly different protein concentrations that were used in the assay to correct for enterokinase activatable enzyme activity. In the presence of 1 mM EDTA, however, the Cys-122 trypsinogen autoactivated much more rapidly at pH 8.0, whereas autoactivation was found to proceed more or less at the same rate compared with wild-type trypsinogen at pH 5.0 (Fig. 4, *E* and *F*).

Experiments that were performed to study the activation of trypsinogen by human cathepsin B resulted in similar enzyme kinetics as seen for enterokinase activation of trypsinogen, *i.e.* when equal protein amounts of wild-type and Cys-122 trypsinogen were activated with cathepsin B, the activation of the Cys-122 mutant was found to be dramatically reduced and only when the amounts of protein were corrected for enterokinase activatable (potential) activity did the mutant activate marginally faster (Fig. 5*A*). These results were independent of whether or not calcium was present or chelated with EDTA (data not shown). Another property of trypsin that could po-

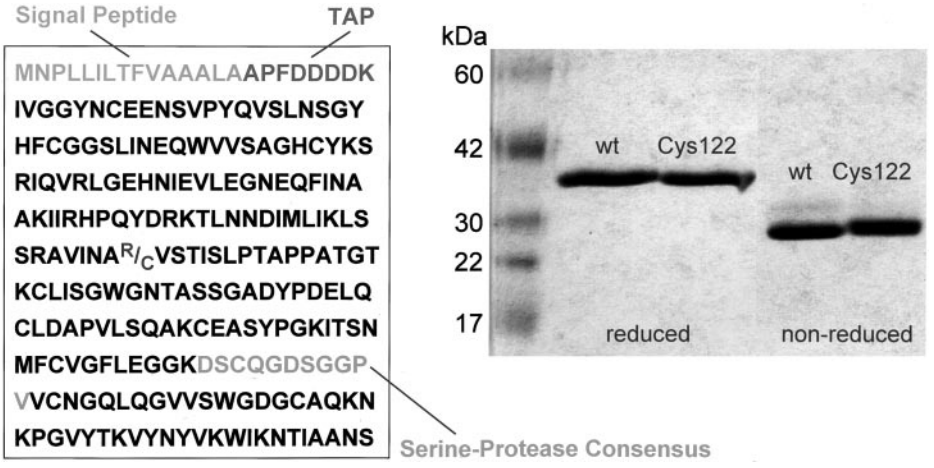

FIG. 3. **Protein sequence of recombinant human PRSS1 into which an arginine to cysteine mutation was introduced by site-directed mutagenesis at codon 122.** The corresponding, Coomassie-stained SDS gels under reducing and nonreducing conditions are shown on the *right*. Note that the Cys-122 trypsin runs somewhat slower through the gel under nonreducing conditions.

TABLE I
Kinetic parameters of wild-type and Cys-122 trypsin on synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide at 22 °C
Data indicate means ± S.E. (n = 3).

	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	M	$s^{-1}\ M^{-1}$
Wild-type	90.4 ± 1.1	$2.1 \pm 0.1 \times 10^{-5}$	4.3×10^{6}
Cys-122	33.2 ± 0.5	$2.0 \pm 0.1 \times 10^{-5}$	1.6×10^{6}

tentially confer a gain of function is the kinetic at which the active trypsin is inactivated or undergoes autolysis (15). In the presence of 5 mM Ca^{2+} or at pH 5.0, wild-type and Cys-122 trypsin were both found to be completely stable and did not undergo autolysis (data not shown; for wild type, see Refs. 7 and 8). However, in the presence of EDTA and at pH 8.0, the Cys-122 mutant trypsin was found to be considerably more resistant to autolysis than the wild-type enzyme (Fig. 5*B*).

Molecular Modeling—The structure of the wild-type PRSS1 in the front view (standard view, Fig. 6*A*) and side view (90° rotation along the *y* axis, Fig. 6*B*) shows that Arg-122 is located at a considerable distance from the active site or the activation domain of trypsin and would therefore be unlikely to directly affect its catalytic activity. In both orientations it can be seen in close proximity to the calcium binding loop (70-loop) of trypsin. In view of this location at the exposed back of the molecule, a replacement of Arg-122 by cysteine could not only affect a potential hydrolysis site of trypsin (9, 18) but would also permit the formation of disulfide bonds between two trypsinogen molecules or between trypsinogen and other proteins. The two molecules in the asymmetric unit of the reported PRSS1 structure (18) were superimposed and are shown together with the neighboring residues of Arg-122 within a distance of 8.0 Å in Fig. 6*C*. Both structures are remarkably similar with exception of the arginine side chains at position 122 (*orange* and *purple* in Fig. 6*C*), which show a high temperature factor (>70 $Å^2$). The fact that Arg-122 can exist in two different orientations in the crystal structure (18) is surprising and reflects structural flexibility at this position. A rotamer search shows that alternative orientations of the side chain of Arg-122 could form favorable interactions with neighboring residues (*i.e.* hydrogen bonds to Gly-74 or Asn-84 and a charged hydrogen bond to Glu-82), which, in turn, would affect the conformation of the calcium-binding loop. When Arg-122 is replaced by cysteine (Fig. 6*D*), the formation of hydrogen bonds with the calcium binding loop is reduced. In the wild-type structure, Ca^{2+} could stabilize the conformation of the 70-loop. In the absence of Ca^{2+}, however, the interaction of Arg-122 with its neighboring residues could result in a different conformation of the 70-loop and therefore

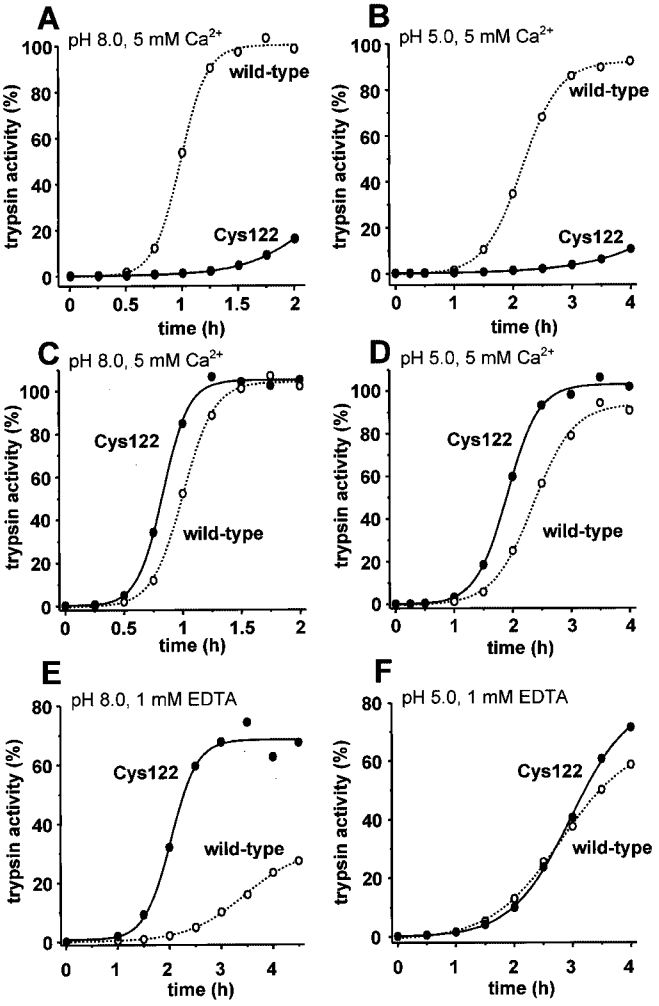

FIG. 4. **Biochemistry of recombinant Cys-122 trypsinogen.** For autoactivation studies of wild-type (*dashed line*) and Cys-122 trypsinogen (*solid line*), equal amounts of purified zymogen (~2.5 μM final concentration) were incubated at 37 °C in 0.1 M Tris-HCl at pH 8.0 (*panel A*) or at pH 5.0 (*panel B*) and in the presence of 5 mM $CaCl_2$ (Ca^{2+}) in a final volume of 100 μl. Aliquots of 2.5 μl were withdrawn from reaction mixtures at the indicated time intervals, and trypsin activity was determined with the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Activity was expressed as a percentage of total wild-type trypsin activity. In *panels C* and *D*, the same experiments (at pH 8.0 in *C*, and at pH 5.0 in *D*) were performed in the presence of 5 mM $CaCl_2$ and in *panels E* (pH 8.0) and *F* (pH 5.0) in the presence of 1 mM EDTA, but the amount of zymogen was corrected for enterokinase activatable trypsin activity; accordingly, the results were expressed as percentage of the potential total activity.

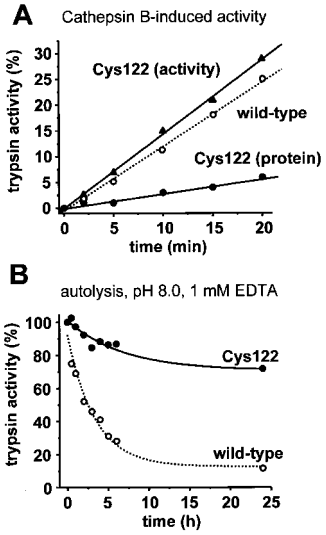

FIG. 5. In the experiments in *panel A*, wild-type trypsinogen (*dashed line*) and Cys-122 trypsinogen (*solid lines*) were activated with human cathepsin B (0.1 M sodium acetate buffer, pH 5.0, 2 mM $CaCl_2$) as described under "Materials and Methods." Cys-122 trypsinogen was either used at equal protein concentrations with wild-type trypsinogen (*solid circles*) or corrected for enterokinase activatable potential activity (*solid triangles*). At the indicated time intervals, 2-μl samples were withdrawn for trypsin activity determination. For autolysis studies in *panel B*, aliquots (final concentration, 2.5 μM) of wild-type trypsinogen (*dashed line*) and Cys-122 trypsinogen (*solid line*) were activated with enterokinase for 60 min at 22 °C in the presence of 5 mM $CaCl_2$ and at pH 8.0, and autocatalytic inactivation of trypsins was followed at 37 °C after addition of 10 mM EDTA (final concentration). Residual activities were expressed as a percentage of trypsin activity measured immediately after enterokinase activation.

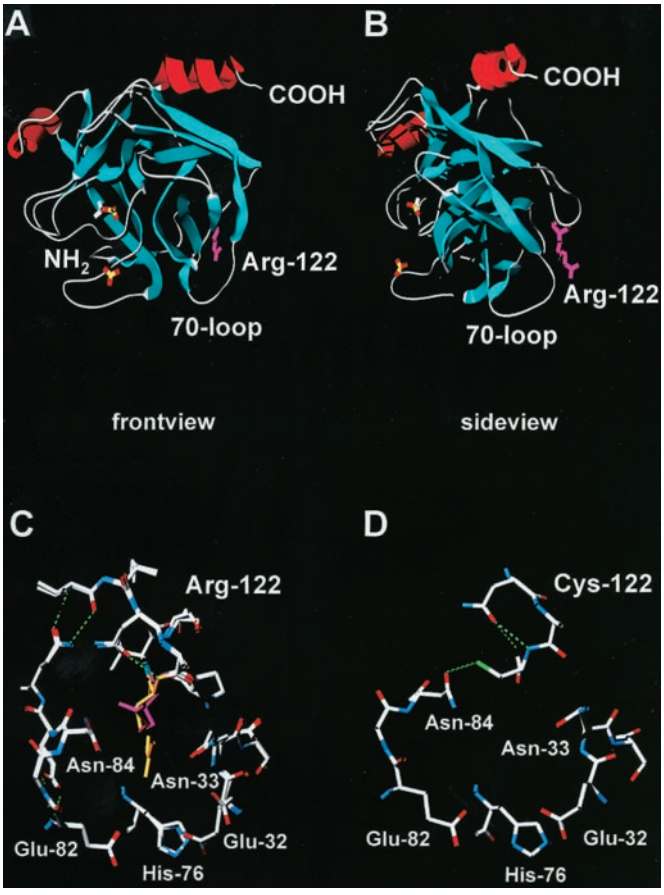

FIG. 6. *A* and *B*, three-dimensional view of the front (*A*) and side (*B*) of human cationic trypsinogen according to the crystallographic structure (18). In relation to the active site and the activation domain, the arginine in codon 122 (*purple*) is found at the opposing surface or back side of the molecule and in close proximity to the 70-loop (calcium-binding loop). The carboxyl terminus and the amino terminus are both indicated, as well as a serine-bound diisopropyl phosphate in the active site (*yellow*) and the phosphorylated tyrosine 151 (*orange*). Helices, *red*; β-strands, *blue*; loops, *white*. *Panel C* shows the different conformations that arginine 122 can adopt in the crystal structures of the asymmetric unit (Ref. 18; *purple* and *orange*). *Panel D* shows that the mutation of Arg-122 to Cys would be predicted to change the hydrogen bonds pattern between the region of codon 122 and the calcium binding loop (70-loop) and potentially affect the conformation of the 70-loop. Oxygen, *red*; nitrogen, *blue*; hydrogen bonds, *green*.

lead to an altered catalytic activity. Cysteine at position 122 could not participate in these predicted interactions, and the conformation of the 70-loop would thus remain unaltered in either the presence or absence of Ca^{2+}.

DISCUSSION

Why structural changes in the cationic trypsinogen gene lead to the onset of hereditary pancreatitis has been a matter of debate. Because pancreatitis has long been regarded as a disease that is caused by proteolytic autodigestion of the pancreas (21) and because trypsin is known to be a potent activator of other pancreatic zymogens in the gut (22), it has been suggested that the trypsinogen mutations that were found in association with hereditary pancreatitis confer a gain of enzymatic function (2, 9). *In vitro* studies have analyzed the biochemistry of recombinant human trypsinogens, into which pancreatitis-associated mutations were introduced and found that, under defined experimental conditions, either a facilitated trypsinogen autoactivation or an extended trypsin activity can result (6–8, 10). Whether these experimental conditions reflect the highly compartmentalized situation under which protease activation begins intracellularly *in vivo* (23, 24) is presently unknown, but the above studies would suggest that either a more effective autoactivation of trypsinogen or an impaired inactivation of trypsin (by degradation or autolysis) would be involved in the onset of hereditary pancreatitis.

A number of arguments, however, have been raised against the gain of trypsin function hypothesis of hereditary pancreatitis. The increased autoactivation of recombinant trypsinogen in *in vitro* studies can be completely abolished by adding physiological concentrations of pancreatic secretory trypsin inhibitor (0.15 M pancreatic secretory trypsin inhibitor, 1 M trypsinogen; Ref. 10) and may therefore have no biological relevance. Statistically, most hereditary disorders are associated with loss of function mutations that render a specific protein either defective or impair its intracellular processing and targeting (25). Moreover, at least five mutations: A16V (26), D22G (6), K23R (27), N29I (28), and R122H (2), have been found in association with hereditary pancreatitis, are located in different regions of the PRSS1 gene, and would thus be expected to have different structural effects on the trypsinogen molecule. It would therefore be easier to explain their common pathophysiology in terms of a loss of enzymatic function rather than through a gain of enzymatic function. In particular, one of these mutations (A16V) also affects the signal peptide cleavage site that is assumed to be involved in the correct processing of trypsinogen (26). Experiments in isolated pancreatic acini and lobules that studied the *in vivo* mechanisms of intracellular zymogen activation have shown that trypsin activity is neither required nor involved in the activation of other digestive proteases and that its most prominent role is in autodegradation (29). This, in turn, would suggest that intracellular trypsin activity has a role in the defense against other, potentially more harmful, digestive proteases and that structural alterations that impair the function of trypsin would eliminate a

protective mechanism rather than generate a triggering event for pancreatitis (30). Whether these experimental observations obtained on rodent pancreatic acini and lobules have any relevance to human hereditary pancreatitis is presently unknown and cannot be readily assumed without further evidence because human cationic trypsinogen has distinct characteristics in terms of its ability to autoactivate and to autodegrade (31, 32).

The kindred reported here is interesting in several respects. The single nucleotide exchange is only one position upstream of the one found in the most common variety of hereditary pancreatitis and leads to an amino acid exchange at the same codon (R122C *versus* R122H). In terms of the diagnostic detection of the R122C mutation in patients suspected of suffering from hereditary pancreatitis, this mutation escapes the conventional screening method with the *Afl*III restriction enzyme digest (2). We therefore propose an alternative screening method using the restriction enzyme *Bst*UI, which has the added advantage of simultaneously detecting the R122H and the R122C mutations, even in the presence of a recently reported neutral polymorphism that makes the R122H mutation inaccessible for screening with the *Afl*III technique (19, 20).

In terms of the clinical manifestation of pancreatitis, the R122C mutation appears to have a milder phenotype than the most common N29I and R122H mutations, as indicated by the relatively late age of initial symptom onset in the two affected patients (with 24 and 52 years, respectively) and the fact that two family members are as yet unaffected carriers. Although the kindred is much too small to make a definitive assessment, the currently known carriers would suggest a disease penetrance of only 50%.

In terms of the question whether hereditary pancreatitis is caused by a gain of trypsin function or a loss of trypsin function, the biochemical characteristics of recombinant Cys-122 trypsinogen are compatible with both possibilities. Only when the amount of trypsinogen in the autoactivation and autolysis assays is corrected for enterokinase activable trypsin activity do the characteristics of Cys-122 trypsinogen resemble those of the previously investigated His-122 mutant (7). In contrast to His-122 trypsinogen, however, the autoactivation of Cys-122 trypsinogen in the absence of Ca^{2+} remains pH-dependent and, at pH 5.0, parallels the autoactivation of wild-type trypsinogen.

Although the differences between Cys-122 and wild-type trypsinogen are unremarkable in the presence of Ca^{2+}, they are significant in the absence of Ca^{2+} and at pH 8.0, where a much more rapid autoactivation and a retarded autolysis can be found. Both the increased autoactivation and the decreased autolysis of Cys-122 trypsin could confer a gain of enzymatic function. Molecular modeling further predicted that the active site of trypsin would remain unaffected by a mutation in codon 122, and, consistent with this prediction, the K_m of Cys-122 trypsin remained unchanged from the wild-type enzyme. The retarded degradation of the active enzyme could be explained by interference with a potential autolysis site, as has also been suggested for His-122 trypsin (9).

When, on the other hand, equal amounts of protein were used for the biochemical studies, the enterokinase-induced activation and the autoactivation of Cys-122 trypsinogen were found to be significantly reduced by 60–70% compared with the wild-type enzyme. The k_{cat} of Cys-122 trypsin, which amounts to only 37% of that of the wild type, suggests that the mutant trypsinogen is largely expressed in a conformation that compromises its activation. Whether this is caused by a destabilization of the interactions between codon 122 and the calcium-binding loop of trypsinogen, as suggested by the modeling studies, or by the formation of mismatched disulfide bonds between the cysteine residues, which would be compatible with the different band appearance in nonreducing SDS gels, remains presently unknown. Interestingly, the activation of Cys-122 trypsinogen by cathepsin B, a mechanism of activation that is not inhibitable by physiological pancreatic secretory trypsin inhibitor concentrations (10), was also dramatically reduced when equal amounts of trypsinogen protein were studied. This observation is of pathophysiological importance because the lysosomal protease cathepsin B is physiologically present in the secretory compartment not only of rodent (33) but also of human pancreas (34) and has been shown to play a crucial role in trypsinogen activation *in vivo* (33, 35).

The interpretation of these changes in terms of their *in vivo* consequences must, by definition, remain speculative. The classical view would be that the R122C mutation largely resembles the R122H mutation and leads to an increased intracellular autoactivation as well as a greatly impaired autolysis and therefore confers a gain of trypsin function. This, in turn, would permit recurrent episodes of proteolytic autodigestion and pancreatitis. In this model the suggested misfolding of Cys-122 trypsinogen would have to be disregarded as a phenomenon that only affects recombinant enzyme *in vitro* and does probably not occur under the compartmentalized intracellular conditions *in vivo*. At most, the misfolding would be considered as a factor that somewhat reduces the gain of enzymatic function and therefore accounts for the milder phenotype in comparison to the R122H mutation, as reflected by the later age of onset and the seemingly lower disease penetrance.

The alternative model would predict that Cys-122 trypsinogen also misfolds or forms mismatched disulfide bridges under intracellular *in vivo* conditions and therefore confers a dramatic loss of trypsin function that cannot be compensated for by the facilitated autoactivation or the impaired autolysis at a basic pH and in the absence of Ca^{2+}. If this scenario should reflect the *in vivo* conditions within the pancreas, it would represent the first direct evidence from a human study for a protective role of trypsin activity in pancreatitis. Short of direct access to living human acini from carriers of PRSS1 mutations or a transgenic animal model into which the human PRSS1 mutations have been introduced, the question of whether the gain of function hypothesis or the loss of function hypothesis correctly predicts the pathophysiology of hereditary pancreatitis cannot presently be resolved.

We conclude that our report of a novel, hereditary pancreatitis-associated mutation in the cationic trypsinogen gene confirms the genetic heterogeneity of the disease (26, 36) and documents the requirement for a novel diagnostic screening approach, which we propose to be the widely applicable restriction enzyme digest with *Bst*UI that makes the inferior *Afl*III method redundant. It further implies that the biochemistry of Cys-122 trypsinogen is compatible with the interpretation that either a gain of enzymatic function or a loss of enzymatic function could represent the triggering mechanism for hereditary pancreatitis.

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Addendum—The timely Internet access to *Journal of Biological Chemistry* manuscripts in press permits other authors to comment on upcoming articles before they appear in print. In this way we were informed not only of an initial report of the R122H polymorphism (19) but also of manuscripts in which two other groups independently report hereditary pancreatitis patients with the R122C mutation (37, 38). This already makes the R122C mutation the fourth most common PRSS1 mutation in patients with hereditary pancreatitis.

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